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Identification of Random Amplified Polymorphic DNA (RAPD) Markers in Endemic Yellow Catfish, *Horabagrus brachysoma* (Gunther 1864)

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Abstract

Random amplified polymorphic DNA (RAPD) analysis was applied to individuals of *Horabagrus brachysoma* sampled from three geographic locations of Western Ghat river systems in India. Of the thirty-two 10-mer RAPD primers screened initially, ten were chosen and used in a comparative analysis of *H. brachysoma* collected from Chalakkudy, Meenachil and Nethravathi river systems. A total of 124 RAPD fragments were amplified, out of which 49 (39.51%) were found to be shared by individuals of all three river systems. The remaining 75 fragments were found to be polymorphic (60.48%). This confirms the suitability of RAPD markers for the study of population genetic structure in yellow catfish stocks.

Introduction

The DNA fingerprinting techniques are extremely efficient for detection of molecular markers that may be utilized in the assessment of

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genetic variation in fish, differentiation of stocks/ populations and hence in fisheries management (Hallermann & Beckmann 1988). The random amplified polymorphic DNA (RAPD) fingerprinting method was first described by Welsh & McClelland (1990) and Williams et al. (1990) in which the amplification of random segments of DNA in the genome is carried out by polymerase chain reaction (PCR) using single primers of arbitrary nucleotide sequence typically with a length of ten nucleotides. They showed that variation of amplified fragments is often inherited in a Mendelian fashion (Bardakci & Skibinski 1994). Since the RAPD technique surveys numerous loci in the genome, it is particularly attractive for analysis of genetic diversity and phylogeny (Clark & Lanigan 1993). Stock identification of several species has been carried out using the above-mentioned techniques (Mamuris et al. 1998; Cagigas et al. 1999; Klinbunga et al. 2000; McCormack & Keegan 2000). This paper is an initial attempt to identify RAPD markers for analyzing the degree of genetic variation in endemic yellow catfish *Horabagrus brachysoma* from three riverine systems along the Western Ghat region of India.

Horabagrus brachysoma is an endemic, yellow catfish belonging to the family Bagridae and is found in rivers originating from the southern part of the biodiversity hotspot Western Ghats (Myers et al. 2000), South India. This species enjoys a good market value as food and ornamental fish (Gopalakrishnan & Ponniah 2000). The species once found in abundance has recorded a sharp decline in the catches due to over-exploitation and is now restricted to a few rivers of Kerala and South Canara viz. Nethravathi, Chalakkudy and Meenachil Rivers. The workshop on Conservation Assessment Management Plan (CAMP) for the evaluation of the status of freshwater species of India held in 1997, categorized this species as endangered based on IUCN criteria due to restricted distribution, loss of habitat, over exploitation, destructive fishing practices and trade (CAMP 1998). Captive breeding and milt cryopreservation techniques have been perfected in this species (Ponniah et al. 2000). The investigation of genetic structure using polymorphic allozyme patterns of this species across its range of natural distribution was carried out by Muneer et al. (2007). However, no information is available on genetic variability using RAPD patterns, hence the RAPD markers are identified.

Materials and Methods

Sampling

Specimens of *H. brachysoma* were obtained through commercial catches from three rivers viz. Meenachil at Kumarakom ($09^0$33′N; $76^0$25'E), Chalakkudy at Kanakkankadavu ($10^0$08'N; $76^0$07'E) and Nethravathi at Kankanadi ($12^0$52'N; $74^0$54'E) (Fig. 1). A total of 90 specimens (30 from each river) were collected for the identification of RAPD markers. The blood samples for DNA extraction were collected from the caudal vein, to avoid contamination, by using heparin (Biological E. LTD, Hyderabad, India) as anticoagulant from the live fish immediately after capture, and stored in 95% ethanol. Only mature fishes were selected from all samples for RAPD analysis.

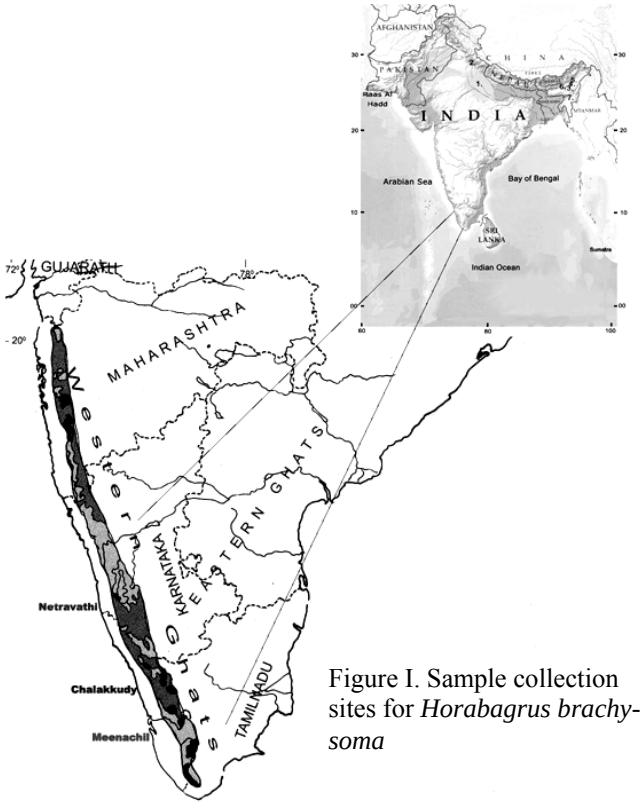

Figure I. Sample collection sites for *Horabagrus brachysoma*

DNA extraction

Total DNA was extracted from the blood samples following the procedures of Taggart et al. (1992) after a few minor modifications. In 50 ml centrifuge tubes, 500 µl of blood (stored in 95% ethanol) from each specimen was taken and the ethanol was decanted by centrifugation at 10000 rpm for 10 min. The blood cells were washed with TE buffer (0.1 M Tris, 0.04 M EDTA, pH 8.0), which was subsequently decanted by centrifugation at 10000 rpm for 10 min. To lysis the cell, incubation buffer (10 mM Tris, 1 mM EDTA, and 0.4M NaCl, 10% SDS and Proteinase K 20mg•ml^{-1}) was added and incubated at 56°C for two hours. After incuba-

tion, the DNA was extracted successively with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (25:1), while centrifuged at 12000 rpm for 15 minutes in each step. The aqueous supernatant was transferred to a fresh tube and $1 \cdot 10^{-1}$ volume of 3M sodium acetate (pH 5.2) was added. The DNA was precipitated with ice cold absolute ethanol. The precipitated DNA was pelleted by centrifugation at 10000 rpm for 15 minutes. After a wash with 70% ethanol, the DNA was vacuum dried and resuspended in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

PCR amplification

Eighty decamer primers (Operon Technologies, Almeda, 4 kits: OPA, OPAA, OPAC and OPAH) were used for this study. From these, 32 primers were selected by detecting the sharp, high intensity, reproducible 6-19 bands using 30 individuals from each population. Out of these 32 primers, 10 primers produced clear cut polymorphic bands among the three populations and these primers were selected as markers (Table 1). The RAPD-PCR reactions were carried out in a PTC 200 thermal cycler (M.J. research, Inc., Watertown, Massachusetts, USA). Amplifications were performed in 25 µl reactions containing 1X reaction buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 5 pmoles of primer, 200 mM dNTPs, 2 U Taq DNA polymerase (Genei, Bangalore, India) and 25 ng of template DNA.

Table 1. Selected primers with concentration and molecular weight for RAPD analysis in *H. brachysoma*

Sl. No:	Primer	Sequences (5'-3')	M.W	Conc. pmoles•µl^{-1}
1	OPA 07	GAAACGGGTG	3108	4.627
2	OPA 09	GGGTAACGCC	3044	5.160
3	OPA 11	CAATCGCCGT	2979	5.533
4	OPA 20	GTTGCGATCC	3018	5.656
5	OPAC 14	GTCGGTTGTC	3041	5.783
6	OPAH 01	TCCGCAACCA	2948	5.413
7	OPAH 02	CACTTCCGCT	2930	6.207
8	OPAH 04	CTCCCCAGAC	2924	5.874
9	OPAH 08	TTCCCGTGCC	2946	6.473
10	OPAH 09	AGAACCGAGG	3077	4.542

To check for DNA contamination, a negative control was set up omitting the DNA from the reaction mixture. The amplification program consisted of an initial denaturation at 95°C for three minutes, followed by 40 cycles of 94°C for 3 minutes, 40°C for 1.30 minutes, 72°C for two

minutes and a final extension at 72°C for 10 minutes. The resulting products were electrophoretically separated in 1.5% agarose gels stained with ethidium bromide (5 μg•ml^{-1}) in TBE buffer (90 mM Tris-borate and 2 mM EDTA, pH 8.0) and the gels were documented using Imagemaster 1D gel documentation system (Amersham Biosciences, USA). The pictures were used for the analysis of the amplified products.

Data analysis

The stained gels were photographed and these photographs were used to analyze the banding patterns i.e. the number of bands was counted as seen in the photographs. A binary matrix was produced whereby the presence or absence of each DNA fragment for each sample was recorded as 1 or 0, respectively. Faint or poorly amplified fragments were excluded from the analysis. Mathematical formulae were used based on a few assumptions. First, all RAPD fragments scored were 2-allele systems i.e. presence (dominant) and absence (recessive) of bands. Second, fragments that migrated at the same position, had the same molecular weight, and were stained to the same intensity, were homologous bands from the same allele, and alleles from different loci did not co-migrate. A third assumption is that both populations fit the Hardy-Weinberg equilibrium, $p^2 + 2pq + q^2 = 1$, with frequencies p (dominant, band present) and q (recessive, band absent) (Clark & Lanigan 1993; Lynch & Milligan 1994). The DNA marker applied along with RAPD samples helped to determine the molecular sizes of the DNA fractions of the fish samples. The RAPD data were analysed by POPGENE Version 1.31 (Yeh et al. 1999).

Results

Genetic variability

A total of 124 different randomly amplified DNA fragments from specimens of *H. brachysoma* were detected consistently with all 10 decamer primers in the three populations. The size of the fragments ranged from 200 to 3000 bp. The number of fragments generated per primer varied from 6 to 19. Of the 124 total RAPD fragments amplified, 49 (39.51%) were found to be shared by individuals of all three populations. The remaining 75 fragments were found to be polymorphic (60.48%). The numbers of fragments amplified and that of polymorphic bands in each primer are given below (Table 2).

Table 2. Performance of the Operon Random Primers in *H. brachysoma*

Primer Code	Meenachil		Chalakkudy		Nethravathi		*Overall populations*	
	Total no. of bands	No. of polymor-phic bands (p%)	Total no. of bands	No. of polymor-phic bands (p%)	Total no. of bands	No. of polymorphic bands (p%)	*Total no. of bands*	*No. of polymor-phic bands (p%)*
OPA-07	8	2 (25.0)	9	5 (55.56)	7	4 (57.14)	9	6 (66.67)
OPA-09	6	1 (16.67)	6	1 (16.67)	8	4 (50.0)	9	6 (66.67)
OPA-11	12	1 (8.33)	12	1 (8.33)	14	2 (14.29)	15	5 (33.33)
OPA-20	10	8 (80.0)	9	3 (33.33)	12	5 (41.67)	12	11 (91.67)
OPAC-14	19	3 (15.79)	17	3 (17.65)	17	1 (5.88)	19	9 (47.37)
OPAH-01	13	9 (69.23)	14	12 (85.71)	14	12 (85.71)	14	12 (85.71)
OPAH-02	12	8 (66.67)	11	8 (72.73)	12	8 (66.67)	13	10 (76.92)
OPAH-04	13	7 (53.85)	13	6 (46.15)	13	5 (38.46)	14	8 (57.14)
OPAH-08	9	2 (22.22)	9	3 (33.33)	8	1 (12.5)	9	3 (33.33)
OPAH-09	10	1 (10.0)	9	0 (0)	6	0 (0)	10	5 (50.0)
Total	112	42 (37.5)	109	42 (38.53)	111	42 (37.84)	124	75 (60.48)

'p%' denotes the percentage of polymorphic bands (in brackets)

Stock- specific markers

Several RAPD fragments show fixed frequencies in a particular population. These can be used as stock specific markers to distinguish the populations. Eight RAPD fragments were obtained as stock specific markers in five primers. These eight fragments were exclusively observed in a particular population (Table 3; Figs. 2 and 3).

Discussion

Random amplified polymorphic DNA (Williams et al. 1990; Welsh & McClelland 1990) is one of the common genetic markers, used for population genetic analysis, pedigree analysis and taxonomic discrimination (Bardakci & Skibinski 1994; Klinbunga et al. 2000; Appleyard & Mather 2002; Callejas & Ochando 2002; Khoo et al. 2002). Several authors have demonstrated that the RAPD-PCR method is a powerful tool in the assessment of discriminating differences at inter-population level in a wide range of organisms including fishes (Welsh & McClelland 1990; Bardakci

& Skibinski 1994; Naish et al. 1995). In the present study, RAPD markers were identified for the analysis of population structure of *H. brachysoma* from three river systems.

Table 3. Stock- specific RAPD markers with size in bp for each population in *H. brachysoma* (Mn: Meenachil, Ch: Chalakkudy and Ne: Nethravathi)

Primer code	Fragment No.	Size (bp)	Mn	Ch	Ne
OPA-09	4th	680	-	-	+
	5th	650	-	-	+
OPA-11	6th	1060	-	-	+
	9th	890	-	-	+
OPA-20	1st	2000	-	-	+
OPAC-14	6th	950	-	-	+
OPAH-02	8th	1010	-	+	-
OPAH-09	3rd	980	+	-	-

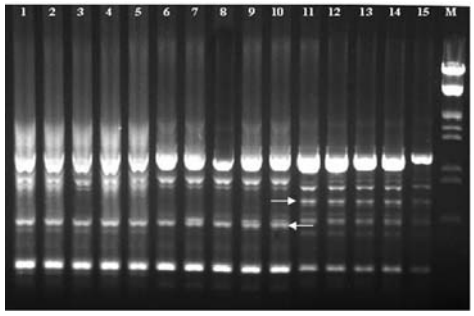

Figure 2. RAPD pattern of *Horabagrus brachysoma* with primer OPA09. Lanes 1-5 Meenachil River; 6-10 Chalakkudy River; 11-15 Nethravathi River; and Lane M- Molecular weight marker-λDNA with *Eco*R1 & *Hind*111 double digest)

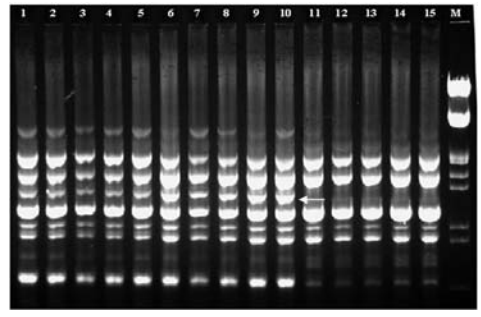

Figure 3. RAPD pattern of *Horabagrus brachysoma* with primer OPAH08. Lanes 1-5 Meenachil River; 6-10 Chalakkudy River; 11-15 Nethravathi River; and Lane M- Molecular weight marker-λDNA with *Eco*R1 & *Hind*111 double digest)

Reproducibility of RAPD markers

Technical problems associated with application of the RAPD technique in the field of genetic population research have been reported by many authors (Hadrys et al. 1992; Lynch & Milligan 1994; Allegrucci et al. 1995; Naish et al. 1995). A disadvantage of this technique is reproducibility of the results (Dinesh et al. 1995; Liu et al 1999). Unreliable products are generated and often the same patterns will not be obtained during the second time even in identical conditions, unless the technique is well standardized. To obtain reproducible results with RAPD, the quality and quantity of the template DNA used is a major key factor (Dinesh et al. 1995).

To standardize the experimental conditions Mamuris et al. (1998) used two different DNA extraction methods, two different polymerases and two thermal cyclers. Taq polymerase purchased from different manufacturers produced similar results when applied on DNA from the same individual in the same thermal cycler. On the contrary, different polymerases as well as different thermal cycles having different temperature cycling profiles produced rather different banding patterns in all individuals screened. In addition, the amplification of DNA obtained by different extraction protocols from the same individual showed slightly different banding patterns, at least after agarose gel electrophoresis (Mamuris et al. 1998). Thus, even if reproducibility of RAPD markers can be obtained in a single laboratory, this seems difficult for different laboratories, unless all conditions are identical. A possible implication of such differences is that qualitative comparisons of data produced by different laboratories, working on the same organism with identical primers would be meaningless, especially when the method is applied to assess specific markers between populations (Mamuris et al. 1998).

In the present study, RAPD analysis was carried out with DNA template extracted from several specimens from three different locations at different times. The DNA polymerase (Taq polymerase), buffer and dNTPs used were from the same source and PCR and electrophoresis were carried out at different intervals. The template DNA quantity (1 µl per single reaction mix) and concentration were kept uniform across samples. This resulted in a high level of reproducibility and sharpness of RAPD profiles in *H. brachysoma* as reported by Ferguson et al. (1995) in *Salmo salar* and Ferguson & Danzmann (1998) in various fish species. The present study shows that under identical amplification conditions, RAPD profiles for any particular primer-template DNA concentration is highly reproducible over a wide range of template RAPD, as reported in seven other fish species by Dinesh et al. (1995).

Genetic variability in RAPD analysis

The RAPD markers were identified in yellow catfish *H. brachysoma* using 10 polymorphic Operon primers. The number of fragments generated per primer varied from six to 19. In *H. brachysoma,* 10 primers generated a total of 124 fragments, producing an average of 12.4 fragments per primer. Among these fragments, 75 (60.48%) were found to be polymorphic as summarized in table 2. The percentage of polymorphism at intra-population level in *H. brachysoma* was relatively low, but in the pooled sample the percentage was high (60.48%) compared to other spe-

cies. Yoon & Kim (2001) reported a total of 652 and 692 bands from 5 primers in two populations (Kunsan and Yesan) of Korean catfish, *Silurus asotus* and among these 298 (45.7%) were polymorphic to Kunsan population and 282 (40.8%) were polymorphic to Kunsan population and 282 (40.8%) were polymorphic to Yesan population. Chong et al. (2000) reported 42 polymorphic RAPD markers in Malaysian river catfish, *Mystus nemurus*. Liu et al. (1998) reported 462 polymorphic bands, an average of 6.1 bands per primer in the channel catfish, *Ictalurus punctatus* and *I. furcatus*. However, Liu et al. (1998) reported a higher value for percentage polymorphic RAPD loci (61.05%) in the channel catfish, *Ictalurus punctatus* and *I. furcatus*. The higher percentage polymorphism scored with RAPD markers in the present study is probably due to preferential amplification of non-coding repetitive regions of the genome that may elude natural selection (Kazan et al. 1992; Callejas & Ochando 2002).

The size and number of the RAPD-PCR product

The molecular weight of 124 RAPD-PCR fragments in *H. brachysoma* ranged from 200 to 3000 bp. Welsh et al. (1991) reported that the number and size of the fragments generated strictly depended upon the nucleotide sequence of the primer used upon the source of the template DNA, resulting in the genome-specific fingerprints of random DNA fragments. However, there was no correlation between the length of the primers and the number of amplified fragments generated in the present case as reported by Dong & Zhou (1998).

The number of amplified products may be related to the G+C content of the primer and template DNA sequence rather than the primer length (Caetano-Anolles et al. 1991). Dong & Zhou (1998) reported that primers with a higher G+C content generated more amplified products. The G+C content did not vary much in the primers selected for the present study, and hence the number of RAPD fragments also did not exhibit much variation with different Operon decamers.

The RAPD analysis is a rapid and convenient technique to generate useful information on stock structure of a species. Since the RAPD technique is less laborious compared to other fingerprinting methods, it produces results with low statistical error (Naish et al. 1995) and does not require prior knowledge of DNA sequences (Hadrys et al. 1992), it may be a promising method to estimate genetic affinities at nuclear level between populations of fish species. The major drawbacks of RAPD markers are related to dominance (i.e. it is not possible to determine if an individual in homozygote or heterozygote) and reproducibility. Despite the apparent

ease of the RAPD methodology, initial empirical optimizations for a given template primer combination can be time consuming. This is because of several parameters- such as quality of template DNA, components of amplification reaction, amplification conditions, primer sequence or the thermal cycler- which influence the number and size of the RAPD markers (Micheli et al. 1994; Dinesh et al. 1995). Thus, one must be cautious about systematic conclusions based on RAPD analysis alone. On the other hand, the possible analysis with unlimited numbers of primers, each detecting variations at several regions in the genome, provides an advantage for RAPD analysis over other techniques (Appleyard & Mather 2002).

The RAPD profiles in the present study displayed a high degree of polymorphism. This confirms the suitability of RAPD markers for discrimination of yellow catfish stocks. In brief, this study yielded highly reproducible RAPD fingerprints, which proved reliable and useful for the discrimination of *H. brachysoma* from three geographically separated river systems viz. Chalakkudy, Meenachil and Nethravathi of the Western Ghat region. The detailed further study with RAPD markers on population genetic structure is progressing in this species.

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